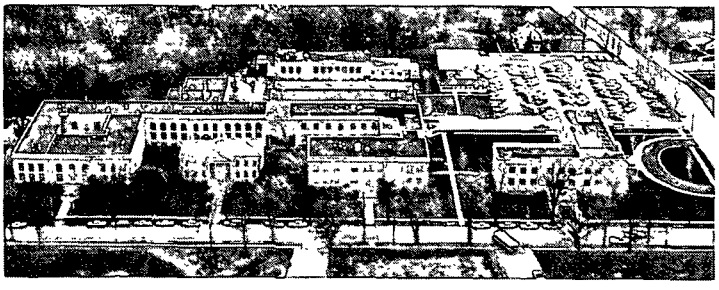

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RECENT PROGRESS IN CONIFER SOMATIC EMBRYOGENESIS RESEARCH

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This publication comprises two papers that were presented at the Fifth International Congress for Plant Tissue and Cell Culture held in Japan, July 10-16, 1982. These presentations contain results of recent research conducted by the conifer tissue culture team at The Institute of Paper Chemistry (Project 3223). The current major goal of this research team is to develop a reliable process for the mass production of somatic embryos from suspension cultures of conifer cells. To this end, the first paper (Verma et al.) relates recent advances in the development of growth media for the conifer cells; the second (Johnson and Carlson) describes a potentially serious impediment to the application to conifer systems of conventional procedures for the induction of somatic embryogenesis.

MEDIA DEVELOPMENT FOR CELL SUSPENSIONS OF CONIFERS

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INTRODUCTION

To date there are no methods available for regenerating plantlets from callus or cell suspension cultures of any of the gymnosperms. Indeed, we are not aware of any other work where a fine suspension culture of conifer cells has been maintained for a reasonably long period of time. One key factor responsible for this slow progress is the lack of a culture medium optimized for sustained growth of conifer tissues and cells *in vitro*. There are reports of plantlet regeneration via organogenesis directly on explants taken from embryonic tissues of a number of coniferous genera (1). These studies have contributed little toward defining nutritional requirements of conifer cells *in vitro*. Herein we describe our studies aimed at media development for generating suspensions from loblolly pine (*Pinus taeda* L.) tissues for the purpose of somatic embryogenesis.

PRELIMINARY MEDIA DEVELOPMENT STUDIES

Broad Spectrum Approach

Having encountered difficulties with woody species, de Fossard conceived this approach to media development (2). When we considered this approach for loblolly pine, the cell size became an important parameter. A loblolly pine cell is ca. 2.5 times the diameter of a wild carrot cell in culture. Larger cells are expected to uptake, metabolize, and store more nutrients; hence, they need a more enriched medium for maintenance *in vitro* than the smaller cells. Therefore, the concentrations of certain ingredients of MS medium (3) were upgraded to various levels arbitrarily (Table 1). Segments of cotyledons from young seedlings were placed on various media in petri dishes. Cultures were incubated in the dark at 23°C. Calli produced were evaluated visually.

Table 1. Media used in the broad spectrum study

Media Components[a]	Concentrations		
	N_1	N_2	N_3
Nitrogen (N)	Same as MS	2 × MS + 10 mM each of glu and allantoin	10 mM each of glu and allantoin + 20 mM K as KCl
Micronutrients (M)	M_1	M_2	M_3
B, Mn	Same as MS	5 × MS	10 × MS
Zn, Fe	Same as MS	3 × MS	5 × MS
Cu, Mo, Co	Same as MS	10 × MS	100 × MS
KI	Same as MS	No change	No change
Growth Regulators (GR) (mg/L)	GR_1	GR_2	GR_3
2,4-D	2.0	5.0	0.0
β-naphthoxyacetic acid	0.0	0.0	5.0
Benzyladenine	0.0	0.0	0.1
NAA			

[a]Macroelements were kept the same as MS, other common ingredients in all treatments included (in mg/L): 2.5 thiamine, 250 inositol, 0.1 pyridoxine HCl, 0.5 nicotinamide and 3000 sucrose, 8000 agar.

Conclusions from this study are as follows: at one month, the greatest variability was attributed to the differences between seedlings; N_3 x M_1 combinations produced maximum calli; M_2 and M_3 were inhibitory; At the end of the third subculture (no explant present), N_3 supported minimum callus, but N_2 x M_2 combinations were judged as the best callus producers. There appeared to be no differences due to various growth regulators. At a later date, callus production decreased even in N_2 x M_2.

These media development efforts were in part thwarted by complications arising from the use of explants. In addition to being genetically different, the explants interacted nutritionally. Our design (Table 1) was also inadequate since macroelements were not varied. Nevertheless, it became apparent that loblolly pine cultures could benefit by appropriate enrichment of media over and above the MS level.

NATURAL MODEL APPROACH TO MEDIA DEVELOPMENT.

The rationale for a novel exercise in media development as pioneered by one of us (JDL) is as follows: "A developing conifer embryo must be influenced by the nutritional environment of the surrounding tissues." Due to lack of space, we cannot go into full details of this Natural Model Approach; however, this information is available elsewhere (4). Based in part on the elemental composition of certain regions of loblolly pine ovules just prior to fertilization, adjustments in the MS medium were made resulting in a new formulation, herein referred to as the loblolly pine medium, or simply LM (Table 2). With LM, the following observations have been made: (a) low Ca in LM facilitates callus proliferation from epidermal and subepidermal layers of cotyledonary explants; (b) on agitation in liquid medium, LM generates fine cell suspensions directly from the original explants such as cotyledons, hypocotyls, or stems of loblolly pine seedlings. These suspensions show sustained growth on subculturing into fresh medium which can be repeated indefinitely. Suspensions can be screened and grown at low inoculum densities in fresh medium (Fig. 1); (c) cell growth and quality are far better than we have seen anytime before; however, our attempts to induce morphogenesis in any of the cultures have so far been unsuccessful.

	COMPOUND	mg/L		COMPOUND	mg/L
a.	NH_4NO_3	1650	c.	$FeSO_4 \cdot 7H_2O$	27.80
	KNO_3	1900		Na_2EDTA	37.30
	$MgSO_4 \cdot 7H_2O$	1850	d.	Sucrose	30,000
	KH_2PO_4	340	e.	myo-Inositol	100
	$CaCl_2 \cdot 2H_2O$	22		Nicotinic acid	0.5
b.	H_3BO_3	31		Pyridoxine HCl	0.1
	$MnSO_4 \cdot H_2O$	21		Thiamine HCl	0.1
	$ZnSO_4 \cdot 7H_2O$	43	f.	β—Naphthoxyacetic acid (NOAA)	5.0
	$Na_2MoO_4 \cdot 2H_2O$	1.25		Benzyladenine	0.1
	$CuSO_4 \cdot 5H_2O$	0.50	or	2,4-Dichlorophenoxyacetic acid (alone)	2.5
	$CoCl_2 \cdot 6H_2O$	0.125			
	KI	4.15			

Table 2. New medium (LM) composition

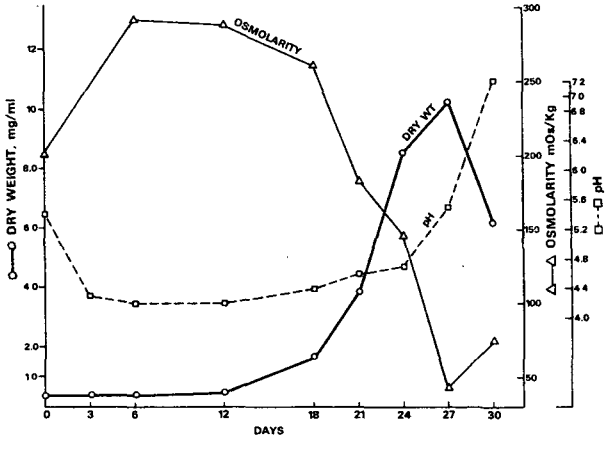

Figure 1. Growth-related changes in loblolly pine cell suspensions grown in LM on roller drums

RELEVANCE OF LM TO WILD CARROT

In LM, wild carrot suspensions grow to greater mass with an accelerated rate than in a wild carrot medium (5). However, LM does not support somatic embryogenesis in wild carrot. Experiments are in progress to find a metabolic explanation of these events.

CONCLUSIONS AND PROSPECTS

A beginning has been made in the natural model approach to media development. This approach is useful and can provide guidelines to the broad spectrum approach on other difficult species, such as the cereals. Insofar as LM is concerned, we believe that further media development efforts must continue to optimize nitrogen, carbon, and phosphorus nutrition. Studies on the wild carrot as the model for experimental embryogenesis are expected to be useful for comparison of metabolic events in coniferous suspensions.

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SOME REDOX CONSIDERATIONS IN CONIFER TISSUE CULTURE

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INTRODUCTION

Glutathione is widely recognized as essential for cell division, although its precise role remains elusive (1,2). It is a significant redox agent in conifers (3), including cultured cells (4). Phenolics also fall in this category when they accumulate under certain culture conditions (5-7). Ozeki and Komamine (8) recently found anthocyanin production to be a good indicator that a portion of a domestic carrot cell population was embryogenic; nevertheless, those specific cell clusters that were accumulating anthocyanin did not undergo somatic embryogenesis when separated from the general population. As reported below and also found by Stafford and Cheng (9) in the case of Douglas-fir cultures, cultured Douglas-fir and loblolly pine cells accumulate colorless proanthocyanidins, close relatives of anthocyanins characteristic of nonembryogenic carrot systems. Comparative temporal analysis of these phenolics and glutathione in cultured conifer cells versus wild carrot cells is presented below and leads to correlations that may further our understanding of somatic embryogenesis possibilities for conifers.

MATERIALS AND METHODS

Pseudotsuga menziesii and Pinus taeda cells were grown in suspension on MS and LM medium, respectively, under light and dark conditions, with and without growth regulators (10). Daucus carota was grown on a wild carrot medium (WCM) (11). Growth regulators used were as follows: Douglas-fir on MS (2.5 ppm 2,4-D, 0.1 ppm BAP); loblolly pine on LM (0.5 ppm 2,4-D); wild carrot on WCM (0.5 ppm 2,4-D). Cells were extracted with 70% acetone to provide suitable aliquots for analyses in triplicate. Analytical procedures for total reductants, reduced glutathione (GSH), and proanthocyanidins were adapted from published procedures (12,13,9). The proanthocyanidin standard was isolated according to Stafford and Cheng (9) from Douglas-fir callus in our laboratories by Stephen Monroe.

RESULTS AND DISCUSSION

We measured separately GSH, proanthocyanidins, and total reductants in cultured cells of wild carrot, loblolly pine, and Douglas-fir as a function of time under embryogenic (for wild carrot) and nonembryogenic conditions, i.e., minus and plus 2,4-D, respectively. It appears that proanthocyanidins may account for much of the nonglutathione reductants in cultured conifer cells whereas wild carrot cells contain no detectable proanthocyanidin but possibly catechin. Anthocyanins are not evident in our wild carrot cultures until after organized embryos have formed in the WCM lacking 2,4-D. While the dilution of 2,4-D leads to embryogenesis in wild carrot, in conifers it leads to enhanced phenolic production and no growth. In fact, in the loblolly pine cell line in Fig. 1, no proanthocyanidin is detectable while the cells grow well in the presence of 2,4-D; however, when the cells are transferred to LM w/o 2,4-D the proanthocyanidin appears and the cells do not grow. In Douglas-fir on MS some phenolics accumulate even in the presence of 2,4-D, with large amounts found in the absence of 2,4-D. In wild carrot cultures (Fig. 1) GSH levels initially fall after subculture into + or - 2,4-D WCM but begin to rise as the cultures emerge from the lag phase. If 2,4-D is absent from the WCM and somatic embryogenesis occurs, this rise is short-lived, reflecting the

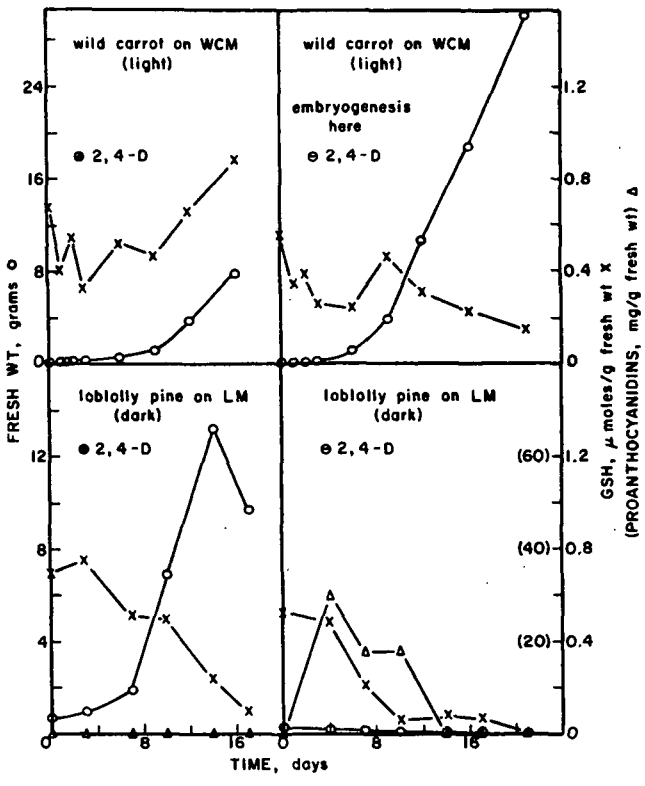

Fig. 1. Temporal changes in fresh weight, proanthocyanidins, and GSH in wild carrot and loblolly pine cultures ± 2,4-D

oxidative processes that accompany organization. However, if 2,4-D is present in the WCM and the culture does not form embryos, the GSH content continues to rise in concert with the proliferative cell division of the unorganized state. Loblolly pine cells do not exhibit these trends in GSH content (Fig. 1), even though the GSH concentrations at time of subculture are similar to those of wild carrot. In the presence of 2,4-D the pine cells maintain a comparable GSH level until well into the linear phase (10 days), but then GSH declines rather than rising. Without 2,4-D the pine cells do not grow in LM and their GSH level falls precipitously after 4 days in culture. Similar data exist for Douglas-fir.

CONCLUSION

It appears that anthocyanin production must follow the organizational events of wild carrot somatic embryogenesis. While conifer cells rarely evidence anthocyanins, they do accumulate colorless proanthocyanidins (particularly in the absence of 2,4-D) which may bear the same relationship to the embryogenesis processes. Accumulation of phenolics may be just a marker of premature cell specialization, or the phenolics may be having direct effects, e.g., by interaction with IAA oxidase (14) or with GSH.

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